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Enhanced Glycerol Content in Wines Made with Immobilized *Candida stellata* Cells

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Screening tests carried out for 10 strains of *Candida stellata* confirmed high levels of glycerol production, although a low fermentation rate and reduced ethanol content were observed. To overcome the poor competition with *Saccharomyces cerevisiae*, fermentation tests with immobilized *C. stellata* cells, alone or in combination with *S. cerevisiae*, have been carried out. The immobilization of *C. stellata* cells consistently reduced the fermentation length when compared with that obtained with free cells, immobilized cells exhibiting about a 30- and a 2-fold improvement in fermentation rate compared with rates for *C. stellata* and *S. cerevisiae* free cells, respectively. Moreover, immobilized *C. stellata* cells produced a twofold increase in ethanol content and a strong reduction in acetaldehyde and acetoin production in comparison with levels for free cells. The evaluation of different combinations of *C. stellata* immobilized cells and *S. cerevisiae* showed interesting results with regard to analytical profiles for practical application in wine making. In fact, analytical profiles of combinations showed, apart from a high glycerol content, a reduction in the amounts of acetic acid and higher alcohols and a consistent increase in succinic acid content in comparison with values for the *S. cerevisiae* control strain. Sequential fermentation first with immobilized *C. stellata* cells and then after 3 days with an added inoculum of *S. cerevisiae* free cells was the best combination, producing 15.10 g of glycerol per liter, i.e., 136% more than the *S. cerevisiae* control strain produced. Fermentation with immobilized *C. stellata* cells could be an interesting process by which to enhance glycerol content in wine.

Glycerol is quantitatively a very important wine constituent. During yeast fermentation, it is the major end product other than ethanol and carbon dioxide. The amount of glycerol formed during fermentation by the yeast species *Saccharomyces cerevisiae* is assumed to be in the range of 7 to 10% of that of ethanol (20), its concentrations in wine varying between 1 and 10 g/liter (16, 17). Occasionally, glycerol may already be present in grape musts infected by molds (14, 19). The amount of glycerol formed is influenced by several factors, such as grape variety, degree of ripeness, fermentation temperature, and yeast strain (16–18). Glycerol is a nonvolatile compound and does not contribute to wine aroma but contributes significantly to the sweetness, with a threshold taste level of 5.2 g/liter in dry white wine (15). Rankine and Bridson (18) have suggested that glycerol contributes significantly to the body and fullness of wines, although a concentration of 25.8 g/liter has been proposed as a level at which an increase in viscosity can be perceived (15). For these reasons, glycerol production is one of the desirable features in wine yeast selection (5).

It was suggested that the glycerol yield in wine can be improved by strain selection (9) and selective hybridization of *S. cerevisiae* wine yeasts (7). Among wine yeasts isolated in winemaking environments, *Candida stellata* is one found largely during the fermentation of musts, contributing significantly to fermentation (10, 11, 13). Moreover, *C. stellata* (formerly known as *Torulopsis bacillaris* and *Torulopsis stellata*) (12) is normally associated with grape berries (22) and particularly with overripe grapes (20). In a previous study of the metabolic behavior of non-*Saccharomyces* wine yeasts (21), high-level production of glycerol by *C. stellata* strains was noted.

The aim of this study was to evaluate the possibility of using *C. stellata* as a fermentation starter to increase the glycerol content in wines.

MATERIALS AND METHODS

Microorganisms. Cultures of *C. stellata* and of *S. cerevisiae* selected for wine making were obtained from the Industrial Yeasts Collection of the Dipartimento di Biologia Vegetale, University of Perugia (DBVPG). Accession numbers were as follows: *S. cerevisiae*, 6663; *C. stellata*, 3175, 3176, 3711, 3827, 3932, 4120, 4121, 4122, 4124, and 4171. All strains were subcultured at 6-month intervals on malt agar medium and maintained at 6°C.

Media. A synthetic grape juice (SGJ) was used in fermentation tests. Each liter of SGJ was composed of three different solutions: solution A (500 ml), solution B (250 ml), and solution C (250 ml). The composition of SGJ was as follows (per liter): solution A, D-glucose, 110 g; D-fructose, 110 g; ergosterol, 10 mg; Tween 80, 1 ml; solution B, L-(+)-tartaric acid, 6.0 g; L-(−)-malic acid, 3.0 g; citric acid, 0.5 g; solution C, YNB (yeast nitrogen base without amino acids and ammonium sulfate) (Difco), 1.7 g; CAA (vitamin-free Casamino Acids) (Difco), 2.0 g; $CaCl_2$, 0.2 g; arginine-HCl, 0.8 g; L-(−)-proline, 1.0 g; L-(−)-tryptophan, 0.1 g. Solutions B and C were buffered at pH 3.5 with NH_4OH and H_3PO_4, respectively. Four milliliters of ergosterol stock solution (Tween 80, 6.25 ml; ergosterol, 62.5 mg in ethanol to make 25 ml) was added to the glucose-fructose solution to complete solution A. The three solutions were sterilized at 121°C (15 lb/in^2) for 20 min separately and then combined aseptically.

YPD (Bacto yeast extract, 10 g/liter, and Bacto Peptone, 10 g/liter [both from Difco], and D-glucose, 50 g/liter) was used for biomass production for immobilized cells.

Screening tests. Screening tests were performed at 25°C in 100-ml Erlenmeyer flasks containing 70 ml of SGJ. One milliliter of 48-h culture at 25°C in the same medium was used to provide an inoculation level of 10^6 cells per ml. After the flasks were aseptically stoppered with special glass valves containing sulfuric acid to allow only CO_2 to escape the system (4), the evolution of fermentation was gravimetrically evaluated by measurement of weight loss due to the amount of carbon dioxide produced.

Tests with immobilized cells. All fermentations were carried out in 1-liter glass minifermentors (containing 500 ml of SGJ under static conditions at 25°C) supplied with two ports, for gas flow and for an inoculum of beads, and a septum

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TABLE 1. Principal enological characteristics of *C. stellata* strains tested[a]

Strain[b]	Ethanol production (% [vol/vol])	Vol. of acidity (g/liter)	Fermentation purity[c]	Fermentation rate (g of CO_2/day)[d]	Glycerol production (g/liter)
3175	5.46	0.10	0.02	0.03	12.3
3176	4.98	0.19	0.04	0.04	11.5
3711	5.16	0.10	0.02	0.04	13.4
3827	5.34	0.10	0.02	0.03	13.8
3932	5.23	0.10	0.02	0.05	11.6
4120	4.82	0.10	0.02	0.03	11.7
4121	5.37	0.10	0.02	0.03	12.2
4122	6.20	0.10	0.02	0.04	11.5
4124	5.07	0.18	0.02	0.03	13.1
4171	5.47	0.11	0.02	0.04	12.5
6663[e]	12.20	1.27	0.10	0.69	6.8

[a] Results are means of values from duplicate experiments. The variation was less than 10%.
[b] Accession numbers are for the DBVPG collection.
[c] Fermentation purity = grams of volatile acidity per liter/percent ethanol (vol/vol).
[d] Time period of 5 days.
[e] Selected wine yeast strain of *S. cerevisiae* used as a control.

of frit glass in order to maintain the beads in the medium and to permit the exit of carbon dioxide.

Cells for immobilization were grown in YPD at 25°C in a rotary shaker (150 rpm) for 72 h (*C. stellata*) and 48 h (*S. cerevisiae*), harvested by centrifugation, washed three times with sterile distilled water, and added to 2.5% Na-alginate (Carlo Erba, Milan, Italy) at a ratio of 5% (wet weight vol) (biomass moisture, 70%; final concentration, 5×10^9 cells per g of beads). By using a peristaltic pump, this mixture was then dripped into $CaCl_2$ (0.1 M) to induce gelation. After 1 h, the beads were washed several times with sterile distilled water and used immediately. The inoculum for immobilized cells was 10^9 cells per ml for *C. stellata* (20% [wt/vol] of the amount of beads in the medium) or 10^8 cells per ml for *S. cerevisiae* (2% [wt/vol] of the amount of beads in the medium). For the inoculum with free cells, yeast cultures were preincubated in SGJ at 25°C for 72 (*C. stellata*) or 48 (*S. cerevisiae*) h and the procedure was standardized to provide an inoculation level of 10^6 cells per ml. The evolution of fermentations was evaluated gravimetrically by the weight loss due to the carbon dioxide evolved. Samples for glycerol analysis (5 ml) collected at different times during the fermentation and final samples were frozen at −20°C after filtration (0.45-μm-pore-size membrane; Millipore Corporation, Bedford, Mass.).

Analyses. Volatile acidity (expressed as grams of acetic acid per liter) was quantified by steam distillation according to official analytical procedures (6). The fermentation rate was evaluated as the amount of carbon dioxide produced after 3 or 5 days of fermentation (grams of CO_2 per day). Ethanol content (expressed as a percentage [vol/vol]) was measured by gas chromatographic analysis (1). Acetaldehyde, ethyl acetate, acetoin, and higher alcohols were detected by gas-liquid chromatography as described by Bertuccioli (2). Glucose and fructose (kit no. 139106), acetic acid (kit no. 148261), glycerol (kit no. 148270), and succinic acid (kit no. 176281) were determined by using specific enzymatic kits (Boehringer, Mannheim, Germany). Fermentation purity was calculated as the amount of volatile acidity formed in relationship to ethanol produced (grams of volatile acidity per liter/percent ethanol [vol/vol]), whereas ethanol yield was expressed as the amount of ethanol (in milliliters) per gram of sugar utilized.

RESULTS AND DISCUSSION

Screening tests. The screening carried out for evaluating the principal enological characteristics confirmed the high-level production of glycerol by *C. stellata* strains compared with the *S. cerevisiae* test strain (Table 1). Unfortunately, this behavior was always associated with a very low fermentation rate and reduced production of ethanol. On the other hand, the fermentation of all *C. stellata* strains was characterized by low-level production of acetic acid, showing a good fermentation purity.

From the above results, the principal limitation, for the use in wine making, of *C. stellata* is the low fermentation rate. In fact, one of the most important features of a yeast starter for wine making is the ability to repress the wild yeast flora always present in the musts. To carry out the fermentation in a nonsterile environment, the yeast starter must possess a suitable fermentation rate; however, the different pitching rates of *C. stellata* starter cells did not significantly increase the fermentation rate (data not shown). This limitation could be overcome by utilizing immobilized yeasts. Among the different advantages in the use of immobilized yeasts, one of the most important is the possibility of using high concentrations of biomass to obtain a high reaction rate, avoiding problems of competition (3).

TABLE 2. Principal enological characteristics of strains used in fermentation tests[a]

Test[b]	Fermentation time (days)	Fermentation rate (g of CO_2/day)[c]	Sugar residue (g/liter)[d]	Sugar utilized (g/liter)[d]	Ethanol produced (ml/liter)	Glycerol produced (g/liter)	Ethanol yield (ml/g)
1	14	3.29	0	217.6	125.6	6.4	0.58
2	32	0.28	138.2	79.4	35.6	12.5	0.45
3	9	7.64	45.2	172.4	67.4	13.9	0.39
4	7	11.57	0	217.6	110.3	10.6	0.51
5	7	19.57	0	217.6	100.8	9.0	0.46
6	8	5.86	0	217.6	98.9	15.1	0.45

[a] Results are means of values from duplicate experiments. The variation was less than 10%.
[b] Modalities of the inoculum were as follows: test 1, free cells of *S. cerevisiae* control strain (10^6 cells per ml); test 2, *C. stellata* free cells (10^6 cells per ml); test 3, immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml); test 4, combination of immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml) and *S. cerevisiae* free cells (10^6 cells per ml); test 5, combination of immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml) and immobilized *S. cerevisiae* cells (2% [wt/vol] of amount of beads; 10^8 cells per ml); test 6, immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml) with addition of *S. cerevisiae* free cells (10^6 cells per ml) after 3 days.
[c] Time period of 3 days.
[d] The amount of sugar at the beginning of the experiment was 217.6 g/liter for all tests.

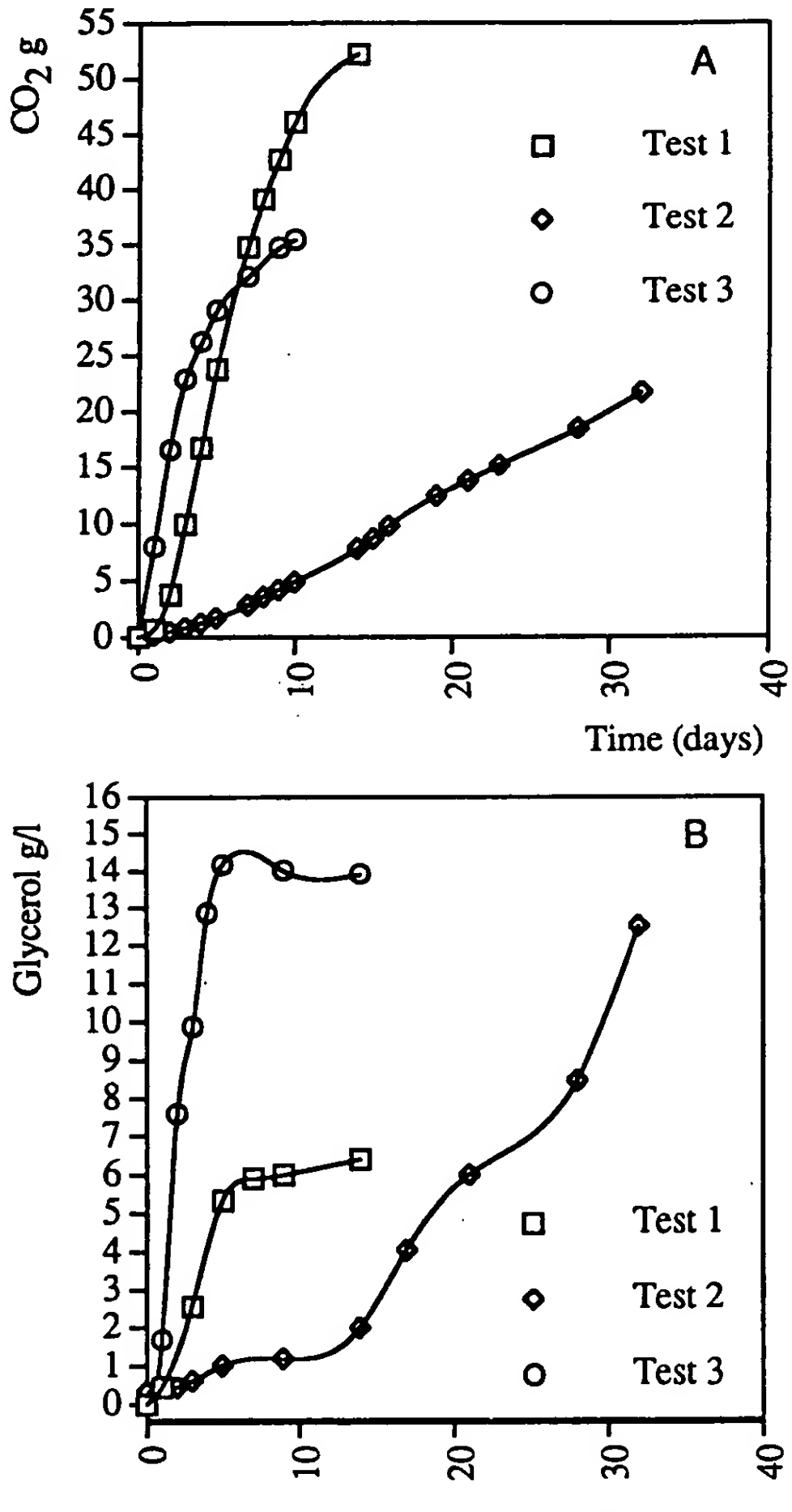

FIG. 1. Kinetics of fermentation (A) and glycerol production (B) by immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml) (test 3), compared with results for free cells of the *S. cerevisiae* control strain (10^6 cells per ml) (test 1) and free cells of *C. stellata* (10^6 cells per ml) (test 2). Each datum point represents the mean of values from duplicate experiments. The variation was less than 10%.

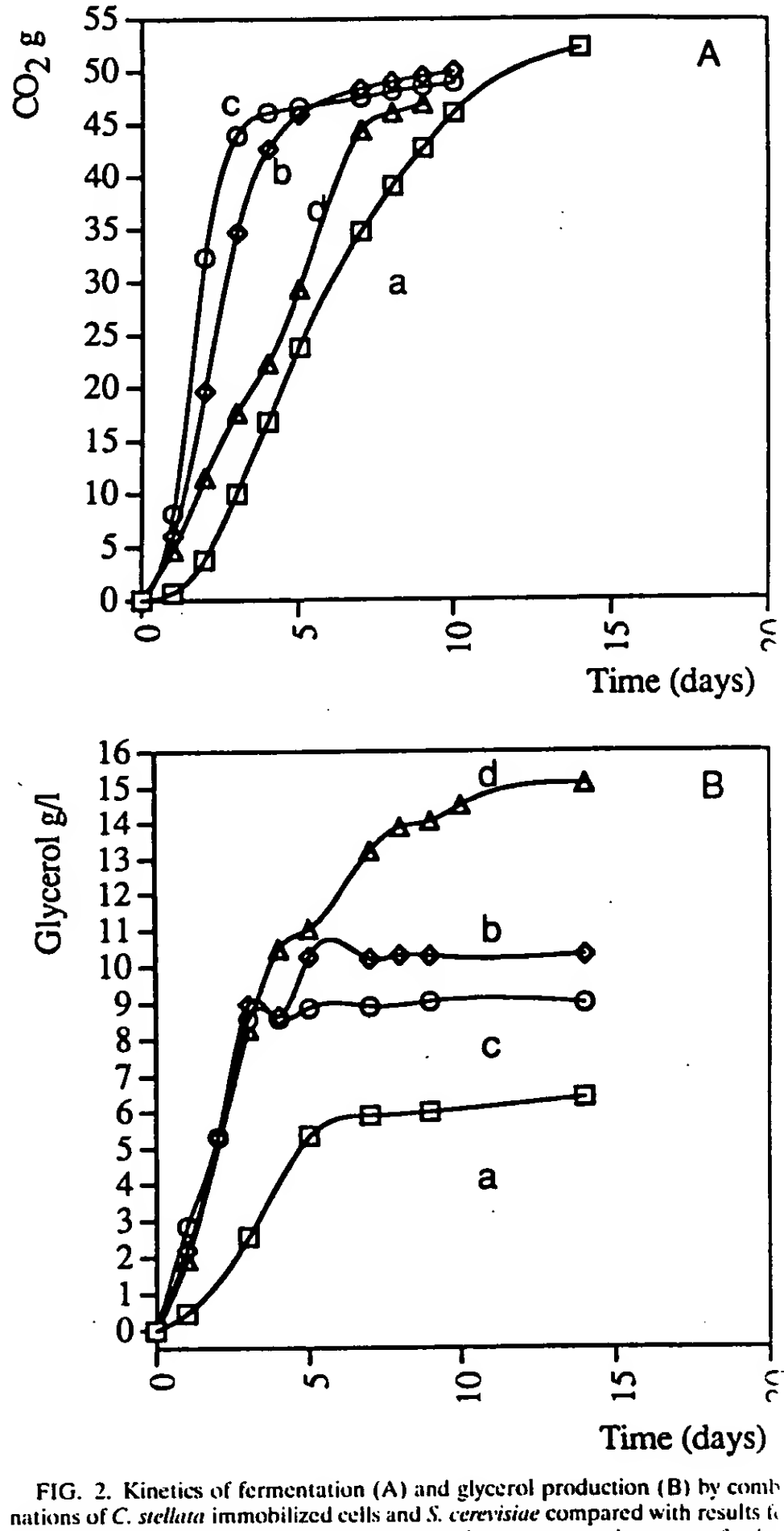

FIG. 2. Kinetics of fermentation (A) and glycerol production (B) by comb nations of *C. stellata* immobilized cells and *S. cerevisiae* compared with results f the *S. cerevisiae* control strain. Each datum point represents the mean of valu from duplicate experiments. The variation was less than 10%. Modalities of t inoculum were as follows: a, free cells of *S. cerevisiae* control strain (10^6 cells p ml); b, *C. stellata* immobilized cells (20% [wt/vol] of amount of beads: 10^9 cel per ml) and *S. cerevisiae* free cells (10^6 cells per ml); c, *C. stellata* immobiliz cells (20% [wt/vol] of amount of beads: 10^9 cells per ml) and *S. cerevisi* immobilized cells (2% [wt/vol] of amount of beads: 10^8 cells per ml); d, *C. stella* immobilized cells (20% [wt/vol] of amount of beads: 10^9 cells per ml) wi addition of *S. cerevisiae* free cells (10^6 cells per ml) after 3 days.

Tests with immobilized cells. To evaluate the use of immobilized *C. stellata* cells to increase the glycerol content in wine, we selected strain DBVPG 3827, especially for its high level of glycerol production, since there was a reduced variability of other enological characteristics among the *C. stellata* strains. A selected wine yeast culture of *S. cerevisiae* DBVPG 6663 (commercial strain Montrachet; Red Star, Milwaukee, Wis.) was used as the control strain.

The influence of the immobilization of *C. stellata* cells is shown in Fig. 1A. The *C. stellata* free cells (test 2) confirmed the low fermentation rate compared with that of the *S. cerev siae* control strain (test 1). As expected, the immobilization c cells (test 3) increased the fermentation rate, resulting in faster rate than that of the *S. cerevisiae* control strain, althoug with a lower level of CO_2 production.

The immobilization procedure caused an increase in th glycerol production rate in accordance with the fermentatio kinetics (Fig. 1B). It is interesting that the *S. cerevisiae* contr strain completed glycerol production during the first stage (

with results for the *S. cerevisiae* control strain.

TABLE 3. Analytical profiles of fermentation tests[a]

Test[b]	Acetic acid (g/liter)	Succinic acid (g/liter)	Acetaldehyde (mg/liter)	Ethyl acetate (mg/liter)	Acetoin (mg/liter)	*n*-Propanol (mg/liter)	2-Methyl-1-propanol (mg/liter)	2-Methyl-1-butanol (mg/liter)	3-Methyl-1-butanol (mg/liter)	Total higher alcohols (mg/liter)
1	1.45	0.32	35.4	15.8	9.0	23.9	172.6	58.0	120.9	375.4
2	0.07	1.23	135.0	0.0	170.4	3.5	39.0	56.3	164.4	263.2
3	0.08	2.53	60.4	0.0	27.1	30.9	42.5	18.0	53.4	144.8
4	0.34	1.32	35.9	27.2	8.4	54.6	57.6	45.6	117.5	275.3
5	0.48	1.20	39.1	15.6	6.1	37.2	47.4	30.9	90.8	206.3
6	0.19	1.63	41.9	14.6	8.4	62.9	47.5	28.6	97.1	236.1

[a] Results are means of values from duplicate experiments. The variation was less than 10%.

[b] Modalities of the inoculum were as follows: test 1, free cells of *S. cerevisiae* control strain (10^6 cells per ml); test 2, *C. stellata* free cells (10^6 cells per ml); test 3, immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml); test 4, combination of immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml) and *S. cerevisiae* free cells (10^6 cells per ml); test 5, combination of immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml) and immobilized *S. cerevisiae* cells (2% [wt/vol] of amount of beads; 10^8 cells per ml); test 6, immobilized *C. stellata* cells (20% [wt/vol] of amount of beads; 10^9 cells per ml) with addition of *S. cerevisiae* free cells (10^6 cells per ml) 3 days later.

days) although the fermentation proceeded until the 14th day, while with *C. stellata* glycerol evolution was in strict accordance with the kinetics of fermentation.

Combinations of *C. stellata* immobilized cells and *S. cerevisiae* (free or immobilized cells) produced an increase in kinetics of fermentation in comparison with the rate for the *S. cerevisiae* control strain, achieving a similar level of CO_2 production (Fig. 2A). Particularly fast was the combination of immobilized *C. stellata* cells and immobilized *S. cerevisiae* cells (test 5). The production of glycerol in combination tests was faster and higher than that of the *S. cerevisiae* control strain (Fig. 2B). The metabolic interaction between immobilized *C. stellata* and *S. cerevisiae* in different combinations affected final glycerol amounts. The effects of the different modalities of the *S. cerevisiae* inoculum can be noted after 3 days. When the inoculum of *S. cerevisiae* was lower or its addition was delayed, the increase in available substrate for *C. stellata* allowed an improvement in glycerol production. The increase in fermentation kinetics of immobilized cells was highlighted by fermentation time and fermentation rate (Table 2). Immobilized *C. stellata* cells (test 3) consistently reduced the period of fermentation performed by free cells and caused about a 30- and a 2-fold improvement in fermentation rate in comparison with rates for *C. stellata* free cells and the *S. cerevisiae* control strain, respectively. In fermentation tests carried out with *C. stellata* (tests 2 and 3) it is possible to observe a sugar residue, since this yeast species is a low-level ethanol producer. With immobilized cells (test 3), this residue was lower than that for free cells (test 2), as confirmed by ethanol production, observed to be twofold higher than for free cells. The combination of immobilized *C. stellata* cells and *S. cerevisiae* (tests 4, 5, and 6) produced an increase in glycerol content and a reduction in the amount of ethanol formed in comparison with levels for the *S. cerevisiae* control strain, as highlighted by the lower ethanol yield. Immobilized *C. stellata* cells inoculated after 3 days with *S. cerevisiae* free cells (test 6) provided the best combination with regard to glycerol production, showing an increase of 136% in comparison with levels obtained with the *S. cerevisiae* control strain.

Estimating the analytical characteristics of fermentation tests (Table 3), it is possible to detect a lower level of acetic acid production and a strong increase in succinic acid production in all fermentation tests carried out with *C. stellata*. Moreover, immobilization of *C. stellata* used alone caused a twofold increase in succinic acid content (tests 2 and 3). The excess of acetaldehyde and acetoin production by *C. stellata* free cells, which is a negative feature in wine making, was reduced by immobilization of cells (test 3). In combination tests (tests 4, 5, and 6), levels of these compounds were further reduced and were very similar to those obtained with the *S. cerevisiae* control strain. With regard to the higher alcohols formed, a lower level of production by *C. stellata* free cells than by the *S. cerevisiae* control strain was noted (tests 1 and 2). Further reduction with immobilized *C. stellata* was noted (tests 3 and 2).

The tests carried out with combinations of immobilized *C. stellata* and free (tests 4 and 6) or immobilized (test 5) *S. cerevisiae* cells showed a positive interaction with regard to analytical profiles. In fact, a comparison between tests done with combinations and with the *S. cerevisiae* control strain (test 1) highlighted a constant reduction in amounts of acetic acid and higher alcohols and an increase in succinic acid production. No significant difference in levels of the other compounds was observed. The results of fermentation tests showed that the immobilization of *C. stellata* cells was able to increase the fermentation rate, improving competition with *S. cerevisiae*. Moreover, under these conditions *C. stellata* increased ethanol production. With regard to the other fermentation by-products of enological interest, the use of immobilized *C. stellata* cells increased succinic acid production and reduced levels of undesirable compounds such as acetaldehyde, acetoin, and higher alcohols. Tests using combinations of *C. stellata* and *S. cerevisiae* (tests 4, 5, and 6) resulted in a considerable improvement in glycerol production by the *S. cerevisiae* control strain (41 to 136%) and interesting analytical profiles without sugar residue. The levels of glycerol reached certainly influence the perceived sweetness (15) and probably influence the body and the fullness (18) of wine. In a previous study (8) of the optimization of combined effects of factors influencing glycerol production in grape juice by *S. cerevisiae*, the maximum level obtained was 6.8 g/liter with 300 ppm of sulfite. Selective hybridization (7) improved the glycerol yield to 108%, achieving a concentration of 10 to 11 g/liter. In the present study, the best conditions (test 6; sequential cultures of immobilized *C. stellata* and *S. cerevisiae*) enhanced glycerol production by 136% in comparison with results for the *S. cerevisiae* control strain.

Thus, fermentation with immobilized *C. stellata* cells in combination with *S. cerevisiae* could be a convenient process by which to enhance glycerol content in wine. However, before using this biotechnology in wine making, it is necessary to optimize the method for using immobilized *C. stellata* cells and to verify their behavior during natural grape must fermentation.

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